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Fungal Genetics and Biology 45 (2008) 1016–1026
FUNGAL GENETICS AND BIOLOGY
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# A fumonisin biosynthetic gene cluster in *Fusarium oxysporum* strain O-1890 and the genetic basis for B versus C fumonisin production

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Received 2 January 2008; accepted 14 February 2008
Available online 23 February 2008

**Abstract**

Most species of *Fusarium* that produce fumonisin mycotoxins produce predominantly B fumonisins (FBs). However, *Fusarium oxysporum* strain O-1890 produces predominantly C fumonisins (FCs). In this study, the nucleotide sequence of the fumonisin biosynthetic gene (*FUM*) cluster in strain O-1890 was determined. The order and orientation of *FUM* genes were the same as in the previously described clusters in *Fusarium verticillioides* and *Fusarium proliferatum*. Coding regions of *F. oxysporum* and *F. verticillioides FUM* genes were 88–92% identical, but regions flanking the clusters did not share significant identity. The *FUM* cluster gene *FUM8* encodes an α-oxoamine synthase, and *fum8* mutants of *F. verticillioides* do not produce fumonisins. Complementation of a *fum8* mutant with the *F. verticillioides FUM8* restored FB production. Complementation with *F. oxysporum FUM8* also restored production, but the fumonisins produced were predominantly FCs. These data indicate that different orthologues of *FUM8* determine whether *Fusarium* produces predominantly FBs or FCs.
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*Keywords:* Fumonisins; Mycotoxin biosynthetic genes; Gene cluster; α-Oxoamine synthase; *Fusarium oxysporum*; *Fusarium verticillioides*

## 1. Introduction

Fumonisins are mycotoxins produced by at least 11 species of the fungus *Fusarium*, including the maize pathogens *Fusarium verticillioides* and *Fusarium proliferatum* (Fotso et al., 2002; Rheeder et al., 2002; Leslie et al., 2004; Desjardins, 2006). Fumonisins are common contaminants of maize and are of concern because of epidemiological associations between ingestion of fumonisin contaminated maize and esophageal cancer and neural tube defects in some human populations (Marasas, 1996; Hendricks, 1999). In addition, fumonisins can cause leukoencephalomalacia in horses, pulmonary edema in swine, and cancer and neural tube defects in experimental rodents (Howard et al., 1999; Marasas et al., 2004).

Fumonisins can be divided into structurally distinct groups, four of which have been designated A, B, C and P fumonisins (Musser and Plattner, 1997). Fumonisins consist of a linear 19 or 20-carbon, polyketide-derived backbone with one nitrogen, 3–4 hydroxyl, two methyl, and two tricarballylic ester functions at positions along the backbone (Fig. 1). A, B, C and P fumonisins differ in structure by differences in the nitrogen function and by the length of the carbon backbone. For example, in B and C fumonisins the nitrogen function is a free amine, in A fumonisins it is an acetylated amine, and in P fumonisins it is a 3-hydroxypyridinium (Musser and Plattner, 1997; Sewram et al., 2005). In B fumonisins (FBs) the backbone is 20 carbon atoms long, whereas in C fumonisins (FCs) it is 19 carbon atoms long (Fig. 1).

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1087-1845/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.fgb.2008.02.004

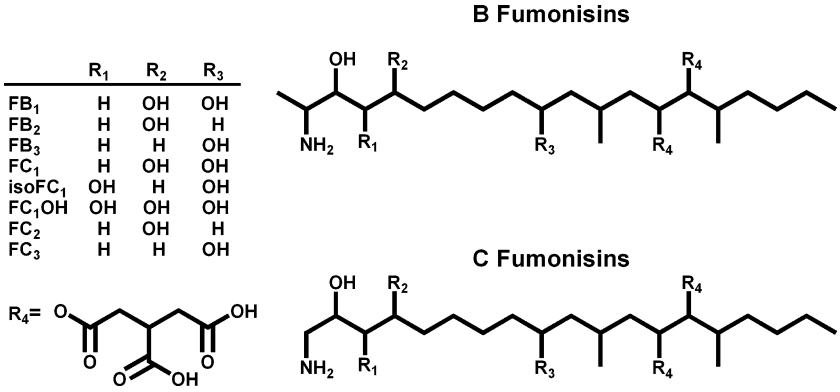


Fig. 1. Chemical structures of B and C fumonisins.

A fumonisin biosynthetic (*FUM*) gene cluster has been described in *F. verticillioides* and *F. proliferatum* (Proctor et al., 2003; Waalwijk et al., 2004). In *F. verticillioides*, the cluster consists of 17 coordinately regulated genes, designated *FUM1–FUM3* and *FUM6–FUM8*, *FUM10*, *FUM11*, and *FUM13–FUM21*. The functions of *FUM1*, *FUM2*, *FUM3*, *FUM7*, *FUM10*, *FUM11*, *FUM13*, *FUM14* and *FUM21* in *F. verticillioides* have been determined genetically by gene disruption and in some cases by mutant complementation (Butchko et al., 2003a,b; Ding et al., 2004; Yi et al., 2005; Butchko et al., 2006; Zaleta-Rivera et al., 2006; Brown et al., 2007). In addition, the functions of *FUM3*, *FUM13* and *FUM14* have been demonstrated biochemically by analysis of the enzymatic activity of heterologously expressed proteins (Ding et al., 2004; Yi et al., 2005; Zaleta-Rivera et al., 2006). Although *fum6* and *fum8* mutants are blocked in fumonisin production, the functions of these genes have not been demonstrated because efforts to identify fumonisin biosynthetic intermediates produced by the mutants have not been successful (Seo et al., 2001; Bojja et al., 2004). Nevertheless, the functions of *FUM6* and *FUM8* have been predicted based on their similarity to genes with known functions combined with the chemical structures of FBs. For example, *FUM8* is highly similar to genes that encode α-oxoamine synthases, a class of enzymes that catalyze the condensation of amino acids and acyl compounds (Christen and Mehta, 2001). This combined with the fact that the FB structure includes an alanine-derived, amine function (Branham and Plattner, 1993a) suggests that the *FUM8* protein catalyzes the condensation of alanine and the fumonisin polyketide (Seo et al., 2001).

Strains of *F. verticillioides* and *F. proliferatum* as well as strains of most other species that have been examined produce predominantly FBs (Musser and Plattner, 1997; Rheeder et al., 2002). Although strains of *F. oxysporum*, which is considered by some to be a species complex (Baayen et al., 2000), do not typically produce fumonisins, there are several reports of fumonisin production by individual strains of this species (Abbas, 1995; Sewram et al., 2005; Seo et al., 1996). Strains of *F. oxysporum* isolated from carnation and asparagus in Korea are highly unusual in that not only do they produce fumonisins, but they produce predominantly FCs rather than FBs (Seo et al., 1996). FC production has been reported in *F. verticillioides*, *F. proliferatum* and some other species but at markedly lower levels than FB production (Branham and Plattner, 1993b; Sewram et al., 2005). In this study, we determined the nucleotide sequence of the *FUM* cluster in an FC-producing strain of *F. oxysporum* and used the sequence to examine whether different *FUM8* orthologues can determine whether FB or FC production predominates in *Fusarium*.

## 2. Materials and methods

### 2.1. Strains and media

The FC-producing strain of *F. oxysporum* used in this study was O-1890 (Seo et al., 1996, 1999). The wild-type, FB-producing strain of *F. verticillioides* used was M-3125 (Leslie et al., 1992). Generation of the fumonisin-nonproducing, *fum8* mutants (strains GfA3245 and GfA3257) of *F. verticillioides* was previously described (Seo et al., 2001). Details on preparation of the following fungal growth media have also been described previously (Tuite, 1969; Seo et al., 2001): V-8 juice agar medium, cracked maize kernel medium, GYEP (0.3% glucose, 0.1% yeast extract, 0.1% peptone), and GYAM (0.24 M Glucose, 0.05% yeast extract, 8.0 mM L-asparagine, 5.0 mM malic acid, 1.7 mM NaCl, 4.4 mM $K_2HPO_4$, 2.0 mM $MgSO_4$, 8.8 mM $CaCl_2$).

### 2.2. Nucleic acid manipulations

In this report, to clearly distinguish between orthologous genes in *F. oxysporum* and *F. verticillioides*, *F. oxysporum* gene designations are preceded by the prefix *Fo* (e.g., *FoFUM8*) and *F. verticillioides* gene designations are preceded by the prefix *Fv* (e.g., *FvFUM8*).

To prepare fungal genomic DNA, strains were grown in liquid GYEP medium. The resulting growth was harvested by vacuum filtration and lyophilized. DNA was extracted from the lyophilized growth with the DNeasy Plant Mini Kit as described by the manufacturer (QIAGEN, Inc.,

Valencia, California). PCR products were purified by agarose gel electrophoresis followed by band purification with the UltraClean DNA Purification kit (MO BIO Laboratories, Inc., Carlsbad, California).

The nucleotide sequences of the *FUM1*, *FUM6*, *FUM7*, *FUM8*, *FUM14*, *FUM15*, *FUM16*, *FUM17*, *FUM19*, *ZFR1*, *ZBD1* and *ORF21* from *F. verticillioides* were used to design PCR primers to amplify internal fragments of orthologous genes in *F. oxysporum* O-1890 using standard primer design and PCR protocols. The *F. oxysporum* amplification products were sequenced directly and the resulting sequence data were used to design primers for the GenomeWalker procedure (Clontech, Inc., Palo Alto, California). In the procedure, *F. oxysporum* O-1890 genomic DNA was digested separately with each of the restriction endonucleases DraI, EcoRV, PvuII and StuI. Each sample of digested DNA was then ligated separately to a DNA-adapter fragment supplied in the GenomeWalker kit. The resulting products of the ligation were then used as templates in nested PCR. In the PCR, one primer was complementary to *F. oxysporum* sequences and the other was complementary to the DNA-adapter sequence. The nucleotide sequence of the resulting PCR products was determined and used to design additional primers to obtain additional sequence data by the GenomeWalker procedure. Primers were also designed to sequence gaps in the data obtained from >2 kb products or to amplify and sequence gaps between GenomeWalker PCR products. In general, more than one GenomeWalker or other PCR product was sequenced to confirm that PCR did not introduce errors into the nucleotide sequences.

*Fusarium oxysporum* nucleotide sequences determination employed the BigDye Terminator v3.1 protocol and a 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). Sequencing primers included both primers that were complementary to *F. oxysporum* DNA and GenomeWalker primer AP2. Initial attempts to sequence a 300-bp region that was located within the *F. oxysporum ZBD1-ORF21* region and that was flanked by a homopolymeric G repeat on one side and homopolymeric A repeat on the other were not successful, presumably because the repeats caused "slippage" of the BigDye polymerase (www.dna.iastate.edu). A subsequent attempt to sequence this region with a primer (5′-CTTTGGAAGTTTTTTTTTTTTG-3′) that included a poly(T) region complementary to the homopolymeric A repeat yielded good quality sequence data, which was then used to design additional primers to obtain the entire sequence between and including the repeats. Sequence data were edited and aligned with the computer program Sequencher version 4.5 (Gene Codes Corporation, Ann Arbor, Michigan). In the GenBank/National Center for Biotechnology Information (NCBI) database, the accession numbers for the DNA sequences of the *F. oxysporum FUM* gene cluster and *ZBD1-ORF21* region are EU449979 and EU477239, respectively. DNA and amino acid sequence comparisons were initially done with the basic local alignment search tool (BLAST) at NCBI. Additional comparisons were done with the alignment functions in DNAMAN version 5.2.0 (Lynnon Corporation, Vaudreuil, Quebec, Canada), BestFit and or PileUp in GCG version 11.1 (Accelrys, Inc., San Diego, California). Predicted amino acid sequences of putative *FUM8* orthologues from *Aspergillus niger* (Accession No. XP_001389110), *Coccidioides immitis* (Accession No. XP_001247436) and *Neosartorya fischeri* (Accession No. XP_001261737) were obtained from the NCBI database.

### 2.3. FUM8 complementation

To confirm the function of *FUM8*, we introduced an *F. verticillioides FUM8* (*FvFUM8*) complementation vector into a *fum8* mutant of the fungus. To construct the *FvFUM8* complementation vector, pFvF8-comp (Fig. 2A), a fragment that spanned from 682 bp upstream of the *FvFUM8* start codon to 863 bp downstream of its stop codon was excised from cosmid clone 4–5 (Proctor et al., 2003) by digestion with KpnI and NheI. The resulting 4481-bp fragment was cloned into KpnI/NheI-digested pT7 Blue-3 (Novagen, Inc., Madison, Wisconsin) to yield plasmid pFvF8-2. A 2.5-kb fragment carrying the geneticin resistance gene (*GENR*) flanked by NotI sites was cloned into pFvF8-2 via the NotI site in the multiple cloning region of pT7 Blue-3 to yield the *FvFUM8* complementation vector pFvF8-comp (Fig. 2A). The *GENR* fragment had been excised from plasmid pGen-Not-1, which was generated by amplifying *GENR* from plasmid pII99 (Tanaka and Tsuge, 2001) with primers rp619 (5′-CATGCGGCCGCATGCCAGTTGTTCCCAGTGATCT-3′) rp620 (5′-CATGCGGCCGCAGAGTAAAGAAGAGGAGCATGTC-3′) to introduce *Not*I sites (underlined in primer sequences) at each end of the gene (Brown et al., 2002). The resulting *GENR* amplicon was cloned into pT7 Blue-3 to yield pGen-Not-1.

To determine whether the function of *FoFUM8* and *FvFUM8* differed, we also introduced a *FoFUM8* complementation vector into *fum8* mutants of *F. verticillioides*. To construct the *FoFUM8* complementation vector, pFoFum8-Gen (Fig. 2A), a fragment that spanned from 15 bp upstream of the *FoFUM8* start codon to 407 bp downstream of its stop codon was amplified from *F. oxysporum* strain O-1890 genomic DNA with primers rp482 (5′-CTTACTAGTGCCCACCATGTCCACACA-3′) and rp483 (5′-TGCATCCTCAAGATACGGATCCAG-3′). A DNA fragment that spanned from 1 to 792 bp upstream of the *FvFUM8* start codon was also amplified from *F. verticillioides* strain M-3125 genomic DNA with primers rp484 (5′-GGTTAGATGATTGATTCCAGGGTACTG-3′) and rp485 (5′-GATGGGCACTAGTAAGGGCTGACACAAGGT-3′). Two to three nucleotides within primers rp482 and rp485 were modified from the original *Fusarium* sequences to introduce a SpeI site (underlined in primer sequences) 12 nucleotides upstream of the *FUM8* start codon. Both amplified fragments were cloned into PCR

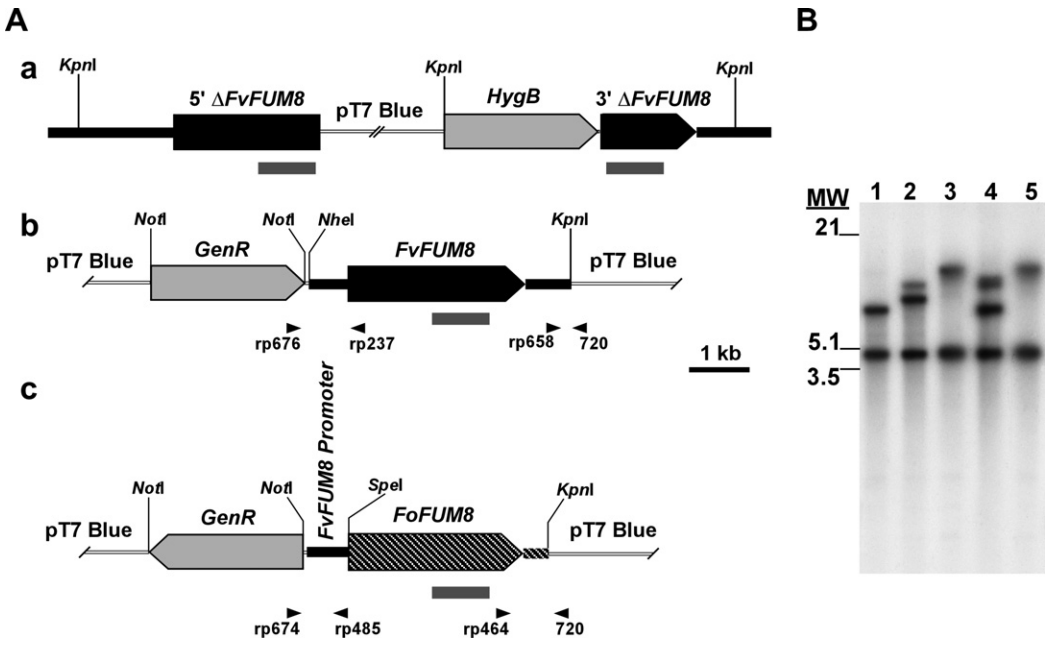


Fig. 2. (A) Structure of the disrupted *FUM8* gene in *fum8* mutant strains GfA3245 and GfA3257 (a), and the structures of the *FvFUM8* (b) and *FoFUM8* (c) complementation vectors (FvF8-comp and FoFUM8-Gen, respectively). The positions of primers used in PCR analysis of transformants are indicated with small black arrows and primer numbers (see text). The dark grey bars indicate the regions corresponding to the hybridization probe used in the Southern analysis. (B) Southern blot analysis of *fum8* mutants of *F. verticillioides* transformed with the *FoFUM8* complementation vector FoFUM8-Gen. Genomic DNA of each strain was digested with KpnI, electrophoresed, transferred to a nylon membrane, and then hybridized to a probe corresponding to a 930-bp region within the *FvFUM8* coding region. Lane 1, progenitor strain GfA3257 (*fum8* mutant); Lanes 2, 3, 4 and 5, *FoFUM8*-complemented strains GmT411, GmT412, GmT418 and GmT403, respectively. MW indicates positions of molecular weight markers; values are kilobase pairs.

cloning vector pT7 Blue-3, and their nucleotide sequences were determined to ensure that PCR amplification did not introduce errors. The two fragments were then spliced together, via the SpeI site introduced on primers rp482 and rp485, to yield plasmid pFoFUM8-4, which consisted of pT7 Blue-3 and 792 bp of the *FvFUM8* 5′-flanking region fused to the entire *FoFUM8* coding region plus 407 bp of the *FoFUM8* 3′-flanking region. The 2.5-kb, NotI-flanked *GENR* fragment from pGen-Not-1 was cloned into pFoFUM8-4 via the NotI site in the multiple cloning region of pT7 Blue-3 to yield the *FoFUM8* complementation vector pFoFUM8-Gen (Fig. 2A).

The two complementation vectors were introduced separately into *F. verticillioides fum8* disruption mutants via the previously described protoplast-mediated transformation procedure (Proctor et al., 1999), and transformants were selected with the antibiotic Geneticin (Sigma-Aldrich Co., St. Louis, Missouri) at 300 μg per ml regeneration medium.

Geneticin-resistant isolates recovered following transformation with either complementation vector were analyzed by PCR with primers designed to amplify fragments that were unique to either the *FvFUM8* or *FoFUM8* complementation vector. Primer pair rp676 (5′-GTTCTATACTCCT ACTTCCAGTTTC-3′) and rp237 (5′-GGTACGTAACG TCTCCCAGGTAGGTAG -3′) and primer pair rp658 (5′-TGTGTGCAGACATCCTCCATAG-3′) and 720 (5′-CA GGACAGGAAACAGCTATGACC-3′) amplified 1000 and 650-bp fragments, respectively, that were unique to the *FvFUM8* vector pFvF8-comp (Fig. 2A). Primer pair rp485 and rp674 (5′-TGAACAGCGACGGTCATGATAACG-3′) and primer pair 720 and rp464 (5′-TCTATAAACCAT GGCTCCACCA-3′) amplified 1010 and 1240-bp fragments, respectively, that were unique to the *FoFUM8* vector pFoFUM8-Gen (Fig. 2A). In addition to PCR, selected pFoFUM8-Gen transformants were analyzed by Southern blot analysis. Genomic DNA was digested with KpnI, subjected to agarose gel electrophoresis, transferred to a Nylon membrane, and then hybridized to a DNA probe corresponding to a 930-bp fragment within the *FvFUM8* coding region (Fig. 2A). The probe also hybridized to the corresponding region of the *FoFUM8* coding region. The hybridization probe was prepared by the Ready-to Go™ DNA labeling kit (Amersham Biosciences, Little Chalfont, Buckinghamshire), and the hybridization procedure followed standard protocols (Sambrook et al., 1989). The probe template was prepared by PCR that employed genomic DNA from wild-type *F. verticillioides* strain M-3125 as a template and oligonucleotides rp224 (5′-CGTAGTAGGAATGAGAAGG ATG-3′) and rp241 (5′-GCAAGCTTTGTGGCTGATT GTC-3′) as primers.

### 2.4. Fumonisin analysis

Strains of *F. verticillioides* and *F. oxysporum* were grown in cracked maize medium and in liquid GYAM medium as previously described (Seo et al., 2001). After a two-week incubation period, cracked maize cultures were extracted

with acetonitrile:water (1:1, v:v) and GYAM cultures were filtered through 0.2 μm Nalgene® (Rochester, USA) filters. Extracts and filtrates were analyzed for the presence of fumonisins by reversed phase liquid chromatography–mass spectrometry (LC–MS) with electrospray by previously described methods (Plattner et al., 1996; Proctor et al., 2006). Fumonisins were quantified by comparison of the integrated intensity of ions corresponding to FBs and FCs to those of fumonisin standards (Plattner et al., 1996).

## 3. Results

### 3.1. Sequence analysis of the FoFUM cluster

Using the GenomeWalker and PCR gap-filling strategies described above, we obtained 53.4 kb of nucleotide sequence from *F. oxysporum* strain O-1890 that included orthologues of the 17 *FUM* genes (Fig. 3) in the same order and orientations as previously described in the *FUM* gene clusters of *F. verticillioides* (GenBank Accession No. AF155773) and *F. proliferatum* (Proctor et al., 2003; Waalwijk et al., 2004; Brown et al., 2005,2007). The *FoFUM* and *FvFUM* gene exons and deduced amino acid sequences are 88–92% and 83–93% identical, respectively (Table 1). The number and positions of introns in the *FoFUM* genes are the same as in the *FvFUM* genes. However, almost all *FoFUM* introns have at least one deletion and or insertion relative to *FvFUM* introns. The intergenic regions in the *FoFUM* and *FvFUM* clusters are 40–77% identical and typically do not differ in length by more than 20%. Exceptions to this are the *FUM10–FUM11* and *FUM21–FUM1* intergenic regions. The *FUM21–FUM1* intergenic region, for example, is 2032 bp in *F. oxysporum* and includes 152 and 48-bp regions that are highly similar to the terminal repeats of a retrotransposon in *F. oxysporum f.* sp. *lycopersici* (Anaya and Roncero, 1995). In contrast, the corresponding region in *F. verticillioides* is only 664 bp and did not include the retrotransposon-like sequences.

The *F. oxysporum* nucleotide sequence data obtained in this study includes 2834 and 3950 bp flanking the *FUM21* and *FUM19* ends, respectively, of the cluster. The first 250 bp immediately 5′ to the *FUM21* start codon is 68% identical in *F. oxysporum* and *F. verticillioides*. However, we did not detect significant identity beyond this 250-bp region. Initial analysis indicated no significant similarity in the *FUM19*-3′ flanking region of *F. oxysporum* and *F. verticillioides* beginning 8-bp downstream of the stop codons. The *FoFUM19*-flanking region includes an open reading frame predicted to encode a cytochrome P450 monooxygenase, tentatively designated as *FoCPM1* (Fig. 3). Subsequent BestFit analyses indicated that a ~230 bp region within *FoCPM1* coding region is 75% identical to nucleotides 7–229 bp immediately downstream of the *FvFUM19* stop codon. Thus, the sequence analysis indicates that the *F. oxysporum FUM19*-flanking region includes a monooxygenase-encoding gene, whereas the corresponding region in *F. verticillioides* includes only a remnant of this gene (Fig. 3). In addition, the results indicate that except for the 222-bp region downstream of *FUM19* and the 250-bp region immediately upstream of *FUM21*,

Table 1
Identities of *FUM* gene orthologues in *F. oxysporum* and *F. verticillioides*

| Gene | Percent identity | |
|---|---|---|
| | Amino acid | Nucleotide[a] |
| FUM1 | 92.0 | 91.1 |
| FUM6 | 92.0 | 90.9 |
| FUM7 | 91.0 | 90.7 |
| FUM8 | 91.0 | 90.1 |
| FUM3 | 92.0 | 91.7 |
| FUM10 | 90.2 | 89.9 |
| FUM11 | 89.1 | 88.4 |
| FUM2 | 93.3 | 87.9 |
| FUM13 | 86.9 | 90.5 |
| FUM14 | 91.0 | 90.4 |
| FUM15 | 88.2 | 91.0 |
| FUM16 | 89.9 | 90.5 |
| FUM17 | 82.9 | 88.7 |
| FUM18 | 84.8 | 88.1 |
| FUM19 | 90.9 | 90.9 |

[a] Values were determined using the nucleotide sequences of coding regions and introns, when present.

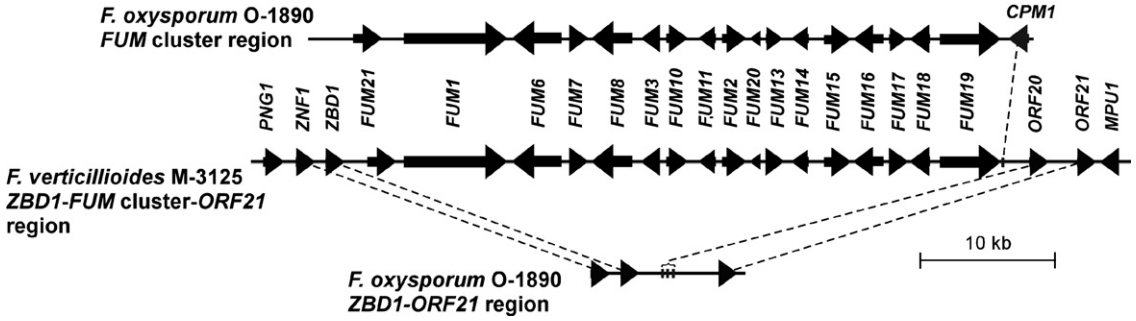


Fig. 3. Comparison of *FUM* clusters and *ZBD1-ORF21* regions in *F. oxysporum* and *F. verticillioides*. In *F. verticillioides* M-3125, the 45.5-kb *FUM* cluster is located between the *ZBD1* and *ORF21*. In *F. oxysporum* O-1890, the *FUM* cluster is not located between *ZBD1* and *ORF21* and may be at a different locus. However, the locations of the *FUM* cluster and the *ZBD1-ORF21* region within the *F. oxysporum* O-1890 genome were not determined. Arrows indicate the relative positions and orientations of gene coding regions. Dashed lines indicate orthologous genes or gene fragments. GenBank accession for the nucleotide sequences of the *FoFUM* cluster and *FoZBD1*/*FoORF21* are EU449979 and EU477239, respectively.

the sequences flanking the ends of the *FUM* clusters in *F. oxysporum* and *F. verticillioides* do not share significant identity.

### 3.2. Sequence analysis of the FoZBD1–FoORF21 region

In *F. verticillioides*, the genes that flank the *FUM* cluster are *ZNF1* and *ZBD1* on the *FUM21* side of the cluster and *ORF20* and *ORF21* on the *FUM19* side (Fig. 3) (Proctor et al., 2003). To determine whether orthologues of these genes were located near the ends of the *FUM* cluster in *F. oxysporum,* we amplified and sequenced *ZBD1* and *ORF21* orthologues and their flanking regions from strain O-1890. With the GenomeWalker and PCR gap-filling protocols, we obtained 10.3 kb of contiguous sequence that spans from the 3′ end of the coding region of a *ZFN1* orthologue to 134 bp downstream of the stop codon of an *ORF21* orthologue (Fig. 3). The *F. oxysporum* sequence includes intact coding regions of *ZBD1* and *ORF21* orthologues. The sequence also includes regions with high levels of identity to *FvORF20*, but these regions are rearranged relative to *F. verticillioides* such that an intact coding region is not present in the *F. oxysporum* sequence. Further analysis of the *F. oxysporum ZBD1–ORF21* intergenic region indicated that it is only 6.7 kb and does not include sequences with significant identity to the *FUM* cluster. Thus, unlike in *F. verticillioides,* in *F. oxysporum* strain O-1890 the *FUM* cluster is not located between *ZBD1* and *ORF21* (Fig. 3).

### 3.3. Fvfum8 mutant complementation

Eleven geneticin-resistant isolates recovered following transformation of *F. verticillioides fum8* mutant strain GfA3245 with *FvFUM8* complementation vector (pFvF8-comp) were examined by PCR to determine if they carried sequence combinations unique to the vector. The 1000 and 650-bp fragments unique to the vector (Fig. 2A) were amplified with primer pairs rp237/rp676 and rp658/720 from genomic DNA of eight of the geneticin-resistant isolates (GmF8C3, GmF8C4, GmF8C9, GmF8C14, GmF8C15, GmF8C16, GmF8C21 and GmF8C23). These results indicate that the eight isolates carried the corresponding regions of the *FvFUM8* complementation vector. Neither fragment was amplified from the three other geneticin-resistant isolates (GmF8C2, GmF8C13 and GmF8C25) examined or from the progenitor strain GfA3245.

Twelve geneticin-resistant isolates recovered following transformation of two *fum8* mutant strains, GfA3245 and GfA3257, with the *FoFUM8* complementation vector (pFoFUM8-Gen) were examined to determine if they carried sequence combinations unique to the vector. Isolates GmT401, GmT402, GmT403, GmT406, GmT408 and GmT410 were recovered from transformation of strain GfA3245, and isolates GmT411, GmT412, GmT413, GmT414, GmT416 and GmT418 were recovered from transformation of strain GfA3257. In PCR analysis with primer pairs rp485/rp674 and rp464/720 the 1010 and 1240-bp fragments unique to the *FoFUM8* vector (Fig. 2A) were amplified from genomic DNA of nine of the geneticin-resistant isolates (GmT401, GmT402, GmT403, GmT408, GmT410, GmT411, GmT412, GmT416 and GmT418). These results indicate that the nine isolates carried the corresponding regions of the *FoFUM8* complementation vector. Neither fragment was amplified from three other isolates (GmT406, GmT413 and GmT414) or from the progenitor strains GfA3245 and GfA3257.

Some of the pFoFUM8-Gen transformants were also analyzed by Southern analysis as described in the Section 2. The hybridization probe was expected to hybridize to duplicated regions of *FUM8* in the progenitor strains GfA3245 and GfA3257 (Fig. 2A) as well as to the corresponding regions of *FoFUM8* in the vector pFoFUM8-Gen. In the analysis, the progenitor strains yielded the expected 4.8 and 7.1-kb bands (Fig. 2B, lane 1). In contrast, transformants that were positive for pFoFUM8-Gen in PCR analysis exhibited three distinct banding patterns, all of which were different than the progenitor pattern. The three patterns suggest that integration of the vector differed among transformants. The first banding pattern (Fig. 2B, lanes 3 and 5) consisted of two bands, a ~5-kb and a ~11-kb band. The 5-kb band was more intense than the ~11-kb band, which is consistent with the 5-kb band consisting of two fragments. Integration of vector pFoFUM8-Gen via homologous recombination into the disrupted *FUM8* locus was expected to yield two 4.9-kb bands and an 11.5-kb band. Thus, the first hybridization pattern is consistent with homologous integration of pFoFUM8-Gen into the disrupted *FUM8* locus. The second banding pattern (Fig. 2B, lane 2) consisted of a ~5, ~8 and ~9-kb band. The absence of the 7.1-kb band observed in the progenitor strains suggests that the vector integrated into the *FUM8* locus. But, differences in this pattern and the first pattern suggested that the integration was accompanied by rearrangement of sequences. Rearrangements of disrupted loci have been previously observed in *Fusarium* (Proctor et al., 1995) and would be facilitated during transformation of *fum8* mutants with the *FoFUM8* complementation vector because of the presence of multiple duplicate sequences (Fig. 2A). Additional Southern and PCR analyses did not definitively resolve to structure of the rearranged locus. However, all analyses were consistent with the presence of the *FoFUM8* coding region in the transformants (data not shown). The third banding pattern exhibited by transformants consisted of three bands, the 4.8- and 7.1-kb bands of the progenitor strains and a ~10-kb band (Fig. 2B, lane 4). This pattern is consistent with integration of the vector at a genomic location other than the *FUM8* locus. Additional Southern analysis that utilized the *FvFUM8* promoter sequence as a hybridization probe was consistent with the above interpretations (data not shown). Thus, PCR and Southern data indicated that

at least some transformants carried the *FoFUM8* complementation vector pFoFUM8-Gen.

HPLC-MS analysis of cracked maize culture extracts of selected transformants revealed that the *fum8* mutant of *F. verticillioides* carrying the *FvFUM8* complementation vector could produce high levels $FB_1$, $FB_2$ and $FB_3$ and in some cases low levels of FCs (Table 2). The kinds and relative levels of FBs and FCs produced by the *FvFUM8*-complemented strains GmF8C-4, GmF8C-9 and GmF8C-21 were similar to those produced by the wild-type progenitor strain (M-3125) of *F. verticillioides* (Table 2, Fig. 4). Analysis of cracked maize culture extracts of selected *fum8* mutants of *F. verticillioides* carrying the *FoFUM8* complementation vector (strains GmT403, GmT411 and GmT412) were also restored to fumonisin production. However, *FoFUM8*-complemented strains produced high levels of $FC_1$, $FC_2$ and $FC_3$ and relatively low levels of $FB_1$, $FB_2$ and $FB_3$ (Fig. 4, Table 2). HPLC-MS analyses of selected transformants grown in GYAM culture yielded essentially the same results as those from cracked maize cultures. However, the levels of fumonisins produced in GYAM were lower than those produced in cracked maize cultures. Only low levels of fumonisin production (<25 μg FBs and FCs combined per g culture) were detected in cultures of the *fum8* mutant strains (GfA3245 and GfA3257) and selected transformants in which the complementation vectors were not detected. These data indicate that complementation of an *F. verticillioides fum8* mutant with *FvFUM8* resulted in predominantly FB production, whereas complementation with *FoFUM8* resulted in predominantly FC production.

Fumonisin production in transformants complemented with the *FoFUM8* vector differed from production in *F. oxysporum* strain O-1890. In strain O-1890, FBs constituted only 0.8% of the total fumonisin production, whereas in *F. verticillioides fum8* mutants complemented with the *FoFUM8* vector, FB production constituted 12–25% of the total fumonisin production (Table 2). In addition, hydroxyl-$FC_1$ ($FC_1OH$) constituted 64% of the total fumonisin ($FC_1$, $FC_1OH$, $FC_2$, $FC_3$, $FB_1$, $FB_2$ and $FB_3$) production by strain O-1890 but constituted only ~1% of

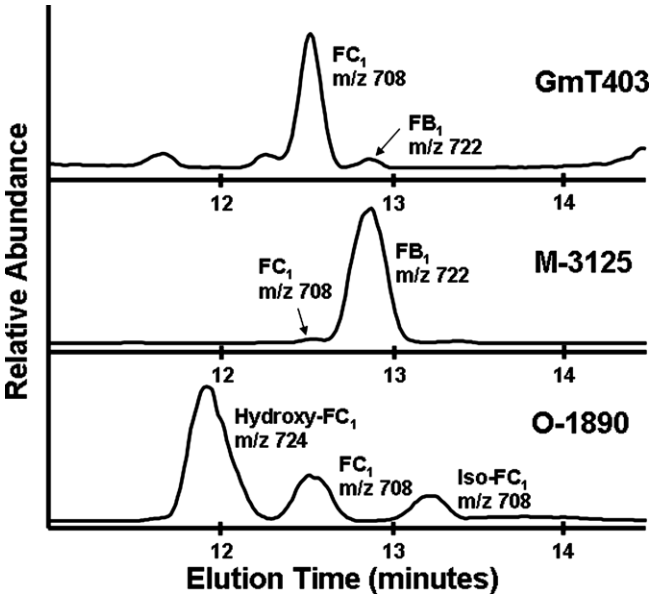


Fig. 4. Selected ion chromatograms from HPLC-MS analysis of acetonitrile:water extracts of *FoFUM8*-complemented *fum8* mutant strain GmT403 (top), wild-type *F. verticillioides* strain M-3125 (middle), and *F. oxysporum* strain O-1890 (bottom). *m*/*z* indicates mass to charge ratio of corresponding peak.

Table 2
FB and FC production in selected transformants recovered following transformation of *F. verticillioides*, *fum8* mutants with the *FvFUM8* or *FoFUM8* complementation vector

| Strain | Genotype[a] | FB[b] concentration (μg $g^{-1}$) | FC[b] concentration (μg $g^{-1}$) | Percent FB[c] | Percent FC[c] |
|---|---|---|---|---|---|
| *F. verticillioides strains* | | | | | |
| M-3125 | *FvFUM8* | 3068 | 41 | 98.7 | 1.3 |
| GmF8C-4 | *Fvfum8*/*FvFUM8* | 1203 | 11 | 99.1 | 0.9 |
| GmF8C-9 | *Fvfum8*/*FvFUM8* | 1174 | 12 | 99 | 1.0 |
| GmF8C-21 | *Fvfum8*/*FvFUM8* | 1112 | 11 | 99 | 1.0 |
| GmT403 | *Fvfum8*/*FoFUM8* | 146 | 1051 | 12.2 | 87.8 |
| GmT411 | *Fvfum8*/*FoFUM8* | 335 | 1009 | 24.9 | 75.1 |
| GmT412 | *Fvfum8*/*FoFUM8* | 163 | 1241 | 11.6 | 88.4 |
| *F. oxysporum strain* | | | | | |
| O-1890 | *FoFUM8* | 8 | 1397 | 0.8 | 99.2 |

[a] *FvFUM8* indicates wild-type *F. verticillioides FUM8*; *FoFUM8* indicates wild-type *F. oxysporum FUM8*; and *Fvfum8* indicates mutated *F. verticillioides FUM8*.

[b] FB values are μg FB1, FB2 and FB3 combined per gram of 2-week-old cracked maize culture. FC values are μg FC1, FC2 and hydroxyl-FC1 combined per gram of 2-week-old cracked maize culture. Total fumonisin (FB + FC) production in *fum8* mutant strains GfA3245 and GfA3257 was <1 μg $g^{-1}$ culture. Wild-type *F. verticillioides* strain M-3125 produced 2315 μg $FB_1$, 558 μg $FB_2$, 195 μg $FB_3$, 0 μg $FC_1OH$, 41 μg $FC_1$, 0 μg $FC_2$ and 0 μg $FC_3$ $g^{-1}$ culture. *F. oxysporum* strain O-1890 produced 8 μg $FB_1$, 0 μg $FB_2$, 0 μg $FB_3$, 710 μg $FC_1OH$, 336 μg $FC_1$, 51 μg $FC_2$, and 52 μg $FC_3$ $g^{-1}$ culture. Strains GmF8C-4, GmF8C-9, GmF8C-21, and other *FvFUM8*-complemented strains produced 335–853 μg $FB_1$, 118–289 μg $FB_2$, 64–223 μg $FB_3$, 0 μg $FC_1OH$, 0–7 μg $FC_1$, 0–2 μg $FC_2$, and 0 μg $FC_3$ $g^{-1}$ culture. Strains GmT403, GmT411, GmT412 and other *FoFUM8*-complemented strains produced 10–215 μg $FB_1$, 3–106 μg $FB_2$, 1–18 μg $FB_3$, 2–20 μg $FC_1OH$, 121–558 μg $FC_1$, 121–675 μg $FC_2$ and 15–85 μg $FC_3$ $g^{-1}$ culture.

[c] Percent values were calculated by first determining the combined levels of FB and FC (FB + FC). The level of FB or FC was then divided by FB + FC and multiplied by 100.

the total production by *FoFUM8*-complemented strains (Table 2).

## 4. Discussion

Most enzyme-encoding genes in *F. verticillioides* that are required for biosynthesis of FBs have been identified and characterized over the past decade. Given their functions, it is likely that all of these genes are also required for production of FCs, A fumonisins (FAs) and P fumonisins (FPs). However, the genetic bases for structural differences between FAs, FBs, FCs and FPs have not yet been elucidated. In the current study, the predominance of FC production by *FoFUM8*-complemented *fum8* mutants of *F. verticillioides* and predominance of FB production by the *FvFUM8*-complemented mutant indicate that different *FUM8* orthologues can determine whether FB or FC production predominates. Thus, the results reported here provide the first evidence for the genetic basis of production of different groups of fumonisins (i.e., FBs and FCs). The results are also consistent with the hypothesis that the *FUM8*-encoded α-oxoamine synthase catalyzes the condensation of an amino acid and a linear polyketide precursor of fumonisins. Precursor feeding experiments indicate that FB biosynthesis involves the condensation of alanine and a linear, 18-carbon polyketide (Branham and Plattner, 1993a). Given their structures, FCs are most likely formed by condensation of glycine and the 18-carbon polyketide (Sewram et al., 2005). Thus, it is likely that the *FvFUM8* α-oxoamine synthase has higher substrate specificity for alanine while the *FoFUM8* enzyme has higher specificity for glycine.

FBs constituted 12–25% of the total fumonisin production in *FoFUM8*-complemented *fum8* mutants of *F. verticillioides*, whereas FBs constituted ~1% of the total fumonisin content in cultures of *F. oxysporum* strain O-1890. This finding suggests that while the *FoFUM8* α-oxoamine synthase has a higher specificity for glycine it can also utilize alanine as a substrate. The finding also indicates that factors other than *FUM8* orthologue can affect the ratio of FBs to FCs produced. One factor could be the proposed interaction of the *FUM1*-encoded polyketide synthase (PKS) and the *FUM8* encoded α-oxoamine synthase (Du et al., 2008). Differences in the *FUM1* PKS in the two species and, therefore, differences in the PKS-oxoamine synthase interaction could contribute to the ratio of FBs and FCs produced during fumonisin biosynthesis. Another factor could be substrate availability. That is, the ratio of FBs to FCs produced could be affected by the levels of alanine and glycine available during fumonisin production. Preliminary attempts to address this possibility have yielded negative results; replacement of L-asparagine in the fumonisin production medium GYAM with L-alanine or L-glycine did not cause detectable changes in the ratios of FBs to FCs produced by either *F. oxysporum* O-1890 or *F. verticillioides* M-3125 (data not shown). It is not clear whether addition of alanine or glycine to GYAM significantly altered levels of these amino acids available for fumonisin production. Therefore, additional experiments are warranted. Such experiments could employ *Fusarium* mutants deficient in alanine or glysine biosynthesis. It may be possible to effectively modulate cellular levels of the amino acids in such mutants by varying their levels in fumonisin production media.

It is not known which differences in the *F. oxysporum* and *F. verticillioides FUM8* protein (Fum8) sequences affect their different amino acid substrate specificities. Residues that affect substrate specificity have been identified in the α-oxoamine synthases 5-aminolevulinic acid synthase (ALAS) and 8-amino-7-oxononanoate synthase (AONS) (Alexeev et al., 1998; Shoolingin-Jordan et al., 2003; Astner et al., 2005). For example, mutations of the threonine at residue 83 (Thr83) markedly altered substrate specificity of the *Rhodobacter sphaeroides* ALAS, which normally exhibits rigid substrate specificity for glycine (Shoolingin-Jordan et al., 2003). Alignment of amino acid sequences of six ALAS and AONS regions with equivalent regions in *Fo*Fum8, *Fv*Fum8 and Fum8 homologues from *A. niger*, *C. immitis* and *N. fischeri* (Fig. 5A) has revealed Fum8 residues equivalent to those that affect substrate specificity or binding in ALASs and or AONSs. Within regions, these residues are the same in all five Fum8 homologues except for the residue equivalent to ALAS Thr83; *Fo*Fum8 has a valine (val579) whereas the other Fum8 orthologues have an alanine at the equivalent position (Fig. 5A). *A. niger* can produce $FB_2$ (Frisvad et al., 2007) and, therefore, the *An*Fum8 substrate is almost certainly alanine. Thus, Fum8 orthologues known to have different substrate specificities differ at a residue equivalent to an ALAS residue (Thr83) that affects specificity. Efforts are underway to determine whether this residue affects substrate specificity of Fum8 orthologues.

The most notable difference between the *F. oxysporum* and *F. verticillioides* clusters was that the DNA flanking the clusters did not share significant identity. In addition, in *F. verticillioides* the 45.5-kb *FUM* cluster is located between *FvZBD1* and *FvORF21* (Fig. 3). In *F. oxysporum* O-1890, there is no evidence for *FUM* gene sequences between the *FoZBD1* and *FvORF21*, which are only ~6.7 kb apart. Waalwijk et al. (2004) noted that the DNA flanking the *FUM21* end of the cluster differs in *F. proliferatum* and *F. verticillioides*. However, they did not have sequence data for the DNA flanking the *FUM19* end of the cluster in *F. proliferatum*. Together, analyses of *FUM* clusters in *F. oxysporum*, *F. proliferatum* and *F. verticillioides* are consistent with the cluster being at different genomic locations in the three species. The different locations of the cluster and the previously reported (Proctor et al., 2004) discontinuous distribution of the *FUM* cluster in species within and closely related to the *Gibberella fujikuroi* complex suggest that the *FUM* cluster has moved within the *Fusarium* genome during the evolution of these species. Furthermore, this movement may have resulted in loss of the cluster in some species. The recent

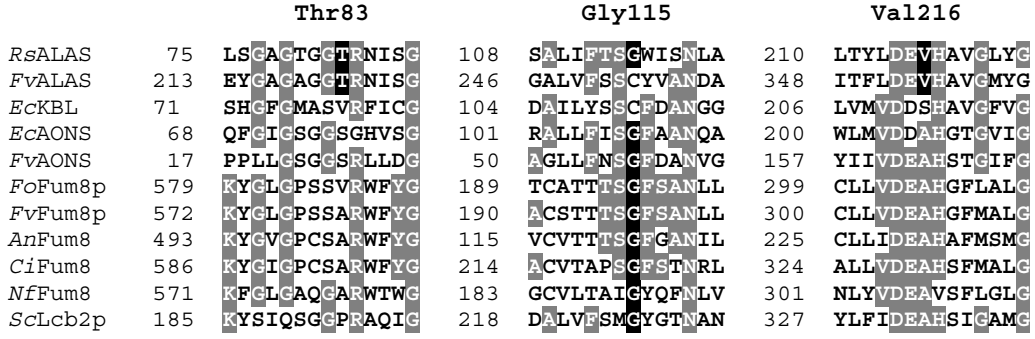


Fig. 5. Alignment of predicted amino acid sequences from three regions of selected α-oxoamine synthases. The aligned regions of ALAS each include a residue (Thr83, Gly115, and Val116—shown in white with a black background) that contributes to amino acid substrate specificity of the enzyme. *Rs*ALAS, *Rhodobacter spheroids* 5-aminolevulinic acid synthase (ABN78836); *Fv*ALAS, putative *F. verticillioides* ALAS orthologue (FVEG_05314); *Ec*KBL, *E. coli* 2-amino-3-ketobutyrate CoA ligase (NP_418074); *Ec*AONS, *Escherichia coli* 8-Amino-7-oxononanoate synthase (NP_415297); *Fv*AONS, putative *F. verticillioides* AONS orthologue (FVEG_06575); *Fo*Fum8, *Fv*Fum8, *Ci*Fum8, *An*Fum8, *Nf*Fum8, the *FUM8* protein orthologues of *F. oxysporum*, *F. verticillioides* (AAG27130), *C. immitis* (XP_001247436), *A. niger* (XP_001389110), and *N. fischeri* (XP_001261737), respectively. Designations in parentheses are accessions for protein sequences. Accessions beginning with FVEG were obtained from the Broad Institute *F. verticillioides* genome sequence database. All other accessions were obtained from NCBI.

finding that the *FUM* cluster is deleted from its position between *ZBD1* and *ORF21* in *F. verticillioides* strains from banana (Van Hove et al., 2006; Glenn et al., 2008) is consistent with this hypothesis.

It remains to be determined whether the cytochrome P450 monooxygenase gene, *FoCPM1*, next to the *FoFUM* cluster functions in fumonisin biosynthesis. $FC_1OH$ is the most abundant fumonisin produced by *F. oxysporum* O-1890 and is structurally identical to $FC_1$ except that $FC_1OH$ has a hydroxyl at carbon atom 3 (C-3) while $FC_1$ does not. Many fungal monooxygenases catalyze hydroxylation reactions. Thus, one possible function of *FoCPM1* is hydroxylation of FCs at C-3. This hypothesis is consistent with the presence of only a 222-bp remnant of *CPM1* next to *FvFUM19* and with the lack of production by *F. verticillioides* of $FB_1$, $FB_2$ and $FB_3$ analogues with a hydroxyl at C-4 (the position equivalent to C-3 in FCs) (Table 2). Whether *FoCPM1* functions in fumonisin biosynthesis could be addressed by disrupting the gene in *F. oxysporum* strain O-1890 and or by heterologous expression of the gene in *F. verticillioides*. The presence of an apparent full-length *FoCPM1* in *F. oxysporum* O-1890 but only a 222-bp remnant of the gene in *F. verticillioides* M-3125 (Fig. 2) suggests that the *FUM19-CPM1* region in *F. oxysporum* is more similar to the ancestral form of this region than the corresponding region in *F. verticillioides*. If *CPM1* does indeed function in hydroxylation of fumonisins, ancestral fumonisins likely included a hydroxyl at the carbon atom equivalent to the C-3 position of FCs.

The *FUM* clusters in *F. oxysporum*, *F. proliferatum* and *F. verticillioides* exhibit relatively little variability; the order and orientation of genes within the clusters are the same, sequence variability of orthologues from different species is relatively low (Proctor et al., 2003; Waalwijk et al., 2004), and, with the possible exception of *CPM1*, there is no evidence for extensive insertions or deletions within individual coding regions that would render genes nonfunctional. This relatively small amount of variability in *FUM* clusters among *Fusarium* species is reflected in relatively small qualitative differences in fumonisin production by different *Fusarium* species that produce these mycotoxins. Although >20 fumonisin analogues have been described, almost all fumonisin-producing species produce predominantly FBs but also tend to produce low levels of FCs and other fumonisins (Seo et al., 1996; Musser and Plattner, 1997; Rheeder et al., 2002; Sewram et al., 2005). The low level variability in *FUM* clusters and fumonisin production in *Fusarium* contrasts the relatively high level of variability in trichothecene biosynthetic gene clusters and trichothecene structural diversity among trichothecene-producing species of *Fusarium* (Brown et al., 2002; Lee et al., 2002; Brown et al., 2003; Peplow et al., 2003; McCormick et al., 2004). Although there is little variability in *FUM* clusters among *Fusarium* species there are marked differences between the *Fusarium* clusters and the *A. niger FUM* clusters. According to Baker (2006), the *A. niger* cluster lacks orthologues of *FUM11*, *FUM12*, *FUM16*, *FUM17* and *FUM18*, the order of *FUM* genes is different, and there are at least three genes in the *A. niger* cluster that are absent in the *Fusarium* clusters. These differences in the *Fusarium* and *A. niger FUM* clusters, however, are not reflected in any marked differences in structural diversity of fumonisin(s) reported to be produced by these fungi (Rheeder et al., 2002; Frisvad et al., 2007). Nevertheless, further comparisons of the *Fusarium* and *A. niger FUM* clusters could provide insight into gene cluster evolution, into the discontinuous occurrence of the cluster within and between fungal genera, and into transcription factor binding sites within the clusters.

## Acknowledgments

We are grateful to Marcie L. Moore and Debora Shane for technical assistance and to Jody Robinson and Jennifer N. Steel at the NCAUR-Core Sequencing Facility for electrophoretic analysis of sequence reactions. We are also

grateful to Daren W. Brown for helpful comments regarding this manuscript. YWL was supported by Grant CG1141 from the Crop Functional Genomics Center of the 21st Century Frontier Research Program.

## References


Abbas, H.K., 1995. First report of fumoinisin $B_1$, $B_2$, and $B_3$ production by *Fusarium oxysporum* var *redolens*. Plant Dis. 79, 968.

Alexeev, D., Alexeeva, M., Baxter, R.L., Campopian, D.J., Webster, S.P., Sawyer, L., 1998. The crystal structure of 8-amino-7-oxononanoate synthase: a bacterial PLP-dependent, acyl-CoA-condensing enzyme. J. Mol. Biol. 284, 401–419.

Anaya, N., Roncero, M.I.G., 1995. *skippy*, a retrotransposon from the fungal plant pathogen *Fusarium oxysporum*. Mol. Gen. Genet. 249, 637–647.

Astner, I., Schulze, J.O., van den Heuvel, J., Jahn, D., Schubert, W.-D., Heinz, D.W., 2005. Crystal structure of 5-aminolevulinate synthase, the first enzyme of heme biosynthesis, and its link to XLSA in humans. EMBO J. 24, 3166–3177.

Baayen, R.P., O'Donnell, K., Bonants, P.J.M., Cigelnik, E., Kroon, L.P.N.M., Roebroeck, E.J.A., Waalwijk, C., 2000. Gene genealogies and AFLP analysis in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae specials causing wilt and rot disease. Phytopathology 90, 891–900.

Baker, S.E., 2006. *Aspergillus niger* genomics: past, present and into the future. Med. Mycol. 44, S17–S21.

Bojja, R.S., Cerny, R.L., Proctor, R.H., Du, L., 2004. Determining the biosynthetic sequence in the early steps of the fumonisin pathway by use of three gene-disruption mutants of *Fusarium verticillioides*. J. Agric. Food Chem. 52, 2855–2860.

Branham, B.E., Plattner, R.D., 1993a. Alanine is a precursor in the biosynthesis of fumonisin $B_1$ by *Fusarium moniliforme*. Mycopathologia 124, 99–104.

Branham, B.E., Plattner, R.D., 1993b. Isolation and characterization of a new fumonisin from liquid cultures of *Fusarium moniliforme*. J. Nat. Prod. 56, 1630–1633.

Brown, D.W., Butchko, R.A.E., Busman, M., Proctor, R.H., 2007. The *Fusarium verticillioides FUM* gene cluster encodes a Zn(II)2Cys6 protein that affects *FUM* gene expression and fumonisin production. Eukaryot. Cell 6, 1210–1218.

Brown, D.W., Cheung, F., Proctor, R.H., Butchko, R.A.E., Zheng, L., Lee, Y., Utterback, T., Smith, S., Feldblyum, T., Glenn, A.E., Plattner, R.D., Kendra, D.F., Town, C.D., Whitelaw, C.A., 2005. Analysis of 87,000 expressed sequence tags reveals alternatively spliced introns in multiple genes of the fumonisin gene cluster. Fungal Genet. Biol. 42, 848–861.

Brown, D.W., McCormick, S.P., Alexander, N.J., Proctor, R.H., Desjardins, A.E., 2002. Inactivation of a cytochrome P-450 is a determinant of trichothecene diversity in *Fusarium* species. Fungal Genet. Biol. 36, 224–233.

Brown, D.W., Proctor, R.H., Dyer, R.B., Plattner, R.D., 2003. Characterization of a *Fusarium* 2-gene cluster involved in trichothecene C-8 modification. J. Agric. Food Chem. 51, 7944–7963.

Butchko, R.A.E., Plattner, R.D., Proctor, R.H., 2003a. *FUM13* encodes a short chain dehydrogenase/reductase required for C-3 carbonyl reduction during fumonisin biosynthesis in *Gibberella moniliformis*. J. Agric. Food Chem. 51, 3000–3006.

Butchko, R.A.E., Plattner, R.D., Proctor, R.H., 2003b. *FUM9* is required for C-5 hydroxylation of fumonisins and complements the meiotically defined *Fum3* locus in *Gibberella moniliformis*. Appl. Environ. Microbiol. 69, 6935–6937.

Butchko, R.A.E., Plattner, R.D., Proctor, R.H., 2006. Deletion analysis of *FUM* genes involved in tricarballylic ester formation during fumonisin biosynthesis. J. Agric. Food Chem. 54, 9398–9404.

Christen, P., Mehta, P.K., 2001. From cofactor to enzymes the molecular evolution of pyridoxal-5′-phosphate-dependent enzymes. Chem. Rec. 1, 436–447.

Desjardins, A.E., 2006. Fusarium Mycotoxins Chemistry, Genetics and Boilogy. APS Press, St. Paul.

Ding, Y., Bojja, R.S., Du, L., 2004. Fum3p, a 2-ketoglutarate-dependent dioxygenase required for C-5 hydroxylation of fumonisins in *Fusarium verticillioides*. Appl. Environ. Microbiol. 70, 1931–1934.

Du, L., Zhu, X., Gerber, R., Huffman, J., Lou, L., Jorgenson, J., Yu, F., Zaleta-Rivera, K., Wang, Q., 2008. Biosynthesis of sphinganine-analog mycotoxins. J. Ind. Microbiol. Biotechnol., in press, doi:10.1007/s10295-008-0361-y.

Fotso, J., Leslie, J.F., Smith, S., 2002. Production of beauvericin, moniliformin, fusaproliferin, and fumonisins $B_1$, $B_2$, and $B_3$ by fifteen ex-type strains of *Fusarium* species. Appl. Environ. Microbiol. 68, 5195–5197.

Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Thrane, U., 2007. Fumonisin $B_2$ production by *Aspergillus niger*. J. Agric. Food Chem. 55, 9727–9732.

Glenn, A.E., Zitomer, N.C., Zimeri, A.M., Williams, L.D., Riley, R.T., Proctor, R.H., 2008. Transformation-mediated complementation of a *FUM* gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. Mol. Plant Microbe Interact. 21, 87–97.

Hendricks, K., 1999. Fumonisins and neural tube defects in south Texas. Epidemiology 10, 198–200.

Howard, P.C., Eppley, R.M., Stack, M.E., Warbritton, A., Voss, K.A., Lorentzen, R.J., Kovach, R., Bucci, T.J., 1999. Carcinogenicity of fumonisin $B_1$ in a two-year bioassay with Fischer 344 rats and B6C3$F_1$ mice. Mycotoxins Suppl. 1999, 45–54.

Lee, T., Han, Y.-K., Kim, K.-H., Yun, S.-H., Lee, Y.-W., 2002. *Tri13* and *Tri7* determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. Appl. Environ. Microbiol. 68, 2148–2154.

Leslie, J.F., Plattner, R.D., Desjardins, A.E., Klittich, C.J.R., 1992. Fumonisin $B_1$ production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium* section Liseola). Phytopathology 82, 341–345.

Leslie, J.F., Zeller, K.A., Logrieco, A., Mulè, G., Moretti, A., Ritieni, A., 2004. Species diversity of and toxin production by *Gibberella fujikuroi* species complex strains isolated from native prairie grasses in Kansas. Appl. Environ. Microbiol. 70, 2254–2262.

Marasas, W.F.O., 1996. Fumonisins: history, world-wide occurrence and impact. In: Jackson, L.S., DeVaries, W., Bullerman, L.B. (Eds.), Fumonisins in Food. Plenum Publishing Co., New York, NY, pp. 1–17.

Marasas, W.F.O., Riley, R.T., Hendricks, K., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martínez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E., Merrill, A.H., 2004. Fumonisins disrupt shingolipid metabolism, folate transport and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J. Nutr. 134, 711–716.

McCormick, S.P., Harris, L.J., Alexander, N.J., Ouellet, T., Saparno, A., Allard, S., Desjardins, A.E., 2004. *Tri1* in *Fusarium graminearum* encodes a P450 oxygenase. Appl. Environ. Microbiol. 70, 2044–2051.

Musser, S.M., Plattner, R.D., 1997. Fumonisin composition in cultures of *Fusarium moniliforme*, *Fusarium proliferatum* and *Fusarium nygami*. J. Agric. Food Chem. 45, 1169–1173.

Peplow, A.W., Meek, I.B., Wiles, M.C., Phillips, T.D., Beremand, M.N., 2003. *Tri16* is required for esterification of position C-8 during trichothecene mycotoxin production by *Fusarium sporotrichioides*. Appl. Environ. Microbiol. 69, 5935–5940.

Plattner, R.D., Weisleder, D., Poling, S.M., 1996. Analytical determination of fumonisins and other metabolites produced by *Fusarium moniliforme* and related species on corn. In: Jackson, L.S., DeVries, J.W., Bullerman, L.B. (Eds.), Fumonisins in Food. Plenum Press, New York, pp. 57–64.

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E., 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic

gene cluster in *Gibberella moniliformis*. Fungal Genet. Biol. 38, 237–249.

Proctor, R.H., Desjardins, A.E., Plattner, R.D., Hohn, T.M., 1999. A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberella fujikuroi* mating population A. Fungal Genet. Biol. 27, 100–112.

Proctor, R.H., Hohn, T.M., McCormick, S.P., 1995. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Mol. Plant Microbe Interact. 8, 593–601.

Proctor, R.H., Plattner, R.D., Brown, D.W., Seo, J.-A., Lee, Y.-W., 2004. Discontinuous distribution of fumonisin biosynthetic genes in the *Gibberella fujikuroi* species complex. Mycol. Res. 108, 815–822.

Proctor, R.H., Plattner, R.D., Desjardins, A.E., Busman, M., Butchko, R.A.E., 2006. Fumonisin production in the maize pathogen *Fusarium verticillioides*: genetic basis of naturally occurring chemical variation. J. Agric. Food Chem. 54, 2424–2430.

Rheeder, J.P., Marasas, W.F.O., Vismer, H.F., 2002. Production of fumonisin analogs by *Fusarium* species. Appl. Environ. Microbiol. 68, 2101–2105.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, New York.

Seo, J.-A., Kim, J.-C., Lee, Y.-W., 1996. Isolation and characterization of two new type C fumonisins produced by *Fusarium oxysporum*. J. Nat. Prod. 59, 1003–1005.

Seo, J.-A., Kim, J.-C., Lee, Y.-W., 1999. *N*-Acetyl derivatives of type C fumonisins produced by *Fusarium oxysporum*. J. Nat. Prod. 62, 355–357.

Seo, J.-A., Proctor, R.H., Plattner, R.D., 2001. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. Fungal Genet. Biol. 34, 155–165.

Sewram, V., Mshicileli, N., Shepard, G.S., Vismer, H.F., Rheeder, J.P., Lee, Y.-W., Leslie, J.F., Marasas, W.F.O., 2005. Production of fumonisin B and C analogues by several *Fusarium* species. J. Agric. Food Chem. 53, 4861–4866.

Shoolingin-Jordan, P.M., Al-Daihan, S., Alexeev, D., Baxter, R.L., Bottomley, S.S., Kahari, I.D., Roy, I., Sarwar, M., Sawyer, L., Wang, S.-F., 2003. 5-Aminolevulinic acid synthase: mechanism, mutations and medicine. Biochim. Biophys. Acta 1647, 361–366.

Tanaka, A., Tsuge, T., 2001. Reporter gene analysis of *AKT3-1* and *AKT3-2* expression during conidial germination of the Japanese pear pathotype of *Alternaria alternata*. J. Gen. Plant Pathol. 67, 15–22.

Tuite, J., 1969. Plant Pathological Methods: Fungi and Bacteria. Burgess Publishing Company, Minneapolis, MN.

Van Hove, F., Waalwijk, C., Gonzalez-Jaen, M.T., Mule, G., Moretti, A., Munaut, F. A 2006. *Fusarium* population from banana missing the fumonisin gene cluster: a new species closely related to *F. verticillioides*? In: Proceedings of MycoGlobe Conference on Advances in genomics, biodiversity and rapid systems for detection of toxigenic fungi and mycotoxins. Monopoli, Italy, p. 98.

Waalwijk, C., van der Lee, T., de Vries, I., Hesselink, T., Arts, J., Kema, G.H.J., 2004. Synteny in toxigenic *Fusarium* species: the fumonisin gene cluster and the mating type region as examples. Eur. J. Plant Pathol. 110, 533–544.

Yi, H., Bojja, R.S., Fu, J., Du, L., 2005. Direct evidence for the function of *FUM13* in 3-ketoreduction of mycotoxin fumonisins in *Fusarium verticillioides*. J. Agric. Food Chem. 53, 5456–5460.

Zaleta-Rivera, K., Xu, C., Yu, F., Butchko, R.A.E., Proctor, R.H., Hidalgo-Lara, M.E., Raza, A., Dussault, P.H., Du, L., 2006. A bidomain nonribosomal peptide synthetase encoded by *FUM14* catalyzes the formation of tricarballylic esters in the biosynthesis of fumonisins. Biochemistry 45, 2561–2569.